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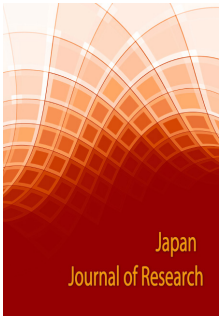

Effects of Photodynamic Therapy on Standard And Fluconazole-Resistant *Candida Albicans* Strains: An *In Vitro* Study

Kedi Jihu[1†], Pugen An[1†], Yizhou Li[2], Xinfei Chen[3], Jizhi Zhao[1*] and Qian Li[1*]

[1]Department of Stomatology, Peking Union Medical College Hospital, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing, 100730, China

[2]Department of Stomatology, Yuquan Hospital, School of Clinical Medicine, Tsinghua University, Beijing 100040, P.R. China,

[3]Department of Clinical Laboratory, Peking Union Medical College Hospital, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing, 100730, China

[†]*These authors contributed equally to this work.*

Correspondence

Prof. Jizhi Zhao & Prof. Qian Li

Department of Stomatology, Peking Union Medical College Hospital, Chinese Academy of Medical Sciences & Peking Union Medical College, No.1 Shuaifuyuan, Wangfujing, Dongcheng District, Beijing, 100730, China

Tel: 86-10-69156332 (JZ); 86-13641278587 (QL)

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Keywords

Candida albicans; Diode laser; Fluconazole resistance; Fungal reduction and antifungal effect of photodynamic therapy; Photodynamic therapy

Abstract

Objective: *The extensive and repetitive use of antifungal drugs has led to resistance from Candida albicans. Photodynamic therapy (PDT) is an emerging and promising approach for combating drug-resistant microbes. The aim of this study was to evaluate photosensitization effects of 810-nm and 670-nm diode lasers against standard suspension of fluconazole-resistant (TS) and susceptible (ATCC90028) C.albicans considering optimum duration of laser irradiation.*

Materials and Methods: *In this in vitro study, suspensions of two kinds of C. albicans were prepared, transferred to 96-holes plates and exposed to 810-nm (500mW, CW) and 670-nm (Periowave®, 200 mW) diode lasers in the presence or absence of methylene blue (MB). 100-fold dilution of the suspension was plated on Sabouraud Dextrose Agar (SDA) and the colony-forming units (CFU) were counted after 24 h of incubation at 37 °C. During irradiation, an infrared thermometer was used to measure the temperature in the experimental well. Also, the morphology of C.albicans was observed microscopically.*

Results: *With MB, both TS and ATCC90028 could be effectively inactivated by 670-nm and 810-nm diode lasers. The antifungal effect of the 670-nm diode laser was almost 100% for 377s; that of the 810-nm laser was 75% for 754s. Within irradiation time 0–390s, the temperature of the fungal suspension changed by <2°C. The laser exposure could not change the drug resistance of the fluconazole-resistant C. albicans. Microscope images suggested that PDT induced C.albicans cells expansion and lysis.*

Conclusion: *PDT using the two types of lasers had excellent sterilization effect on fluconazole-resistant and susceptible C.albicans.*

Introduction

Candida is an opportunistic pathogen that lives in the human oral cavity, skin, and intestinal tract. Normally, it does not cause disease. However, when host immunity is low or patients have taken broad-spectrum antibiotics, *Candida* can cause mucosal or systemic infection[1,2]. Candidiasis is one of the commonest oral infections. Azole antifungal drugs such as fluconazole and itraconazole are the main treatment for *Candida* infection. However, with the extensive use of antifungal drugs, resistant *C. albicans* is becoming increasingly common in the clinic [3]. A 12-month (1 July 2010 through 30 June 2011) laboratory-based surveillance study showed that fluconazole-resistant strains accounted for 6.8%–15% of all *C. albicans* isolates from patients [4]. Treatment of drug-resistant *C. albicans* is challenging for clinicians [5,6].

Photodynamic therapy (PDT) is a selective and noninvasive medical treatment. In previous study, researchers mixed photosensitizers with bacteria and fungi. The photosensitizer adhered to the cell membrane, then, under irradiation at a specific wavelength, the photosensitizer could produce substances that were toxic to the microbial cells, such as singlet oxygen and free radicals [7]. These substances acted on the cell membrane, leading to cell lysis and necrosis [8,9]. The bactericidal effect of PDT has been confirmed [10-14]. However, there have been few studies on application of PDT to *C. albicans*, especially drug-resistant strains. The aim of this study was to evaluate the fungal reduction and antifungal effect of photodynamic therapy of two types of laser on fluconazole-resistant and standard (susceptible) *C. albicans* strains.

Materials and Methods

In our in-vitro study, the two kinds of *C. albicans* (ATCC 90028 and TS) in culture medium (10 µL) was inoculated on Sabouraud dextrose agar plates. After 24h of incubation at 37°C, a suspension of 1 McFarland turbidity (about 3.0×10^6 CFU. ml^{-1}) was prepared with saline. Fungal broth (100 µL) was put into the

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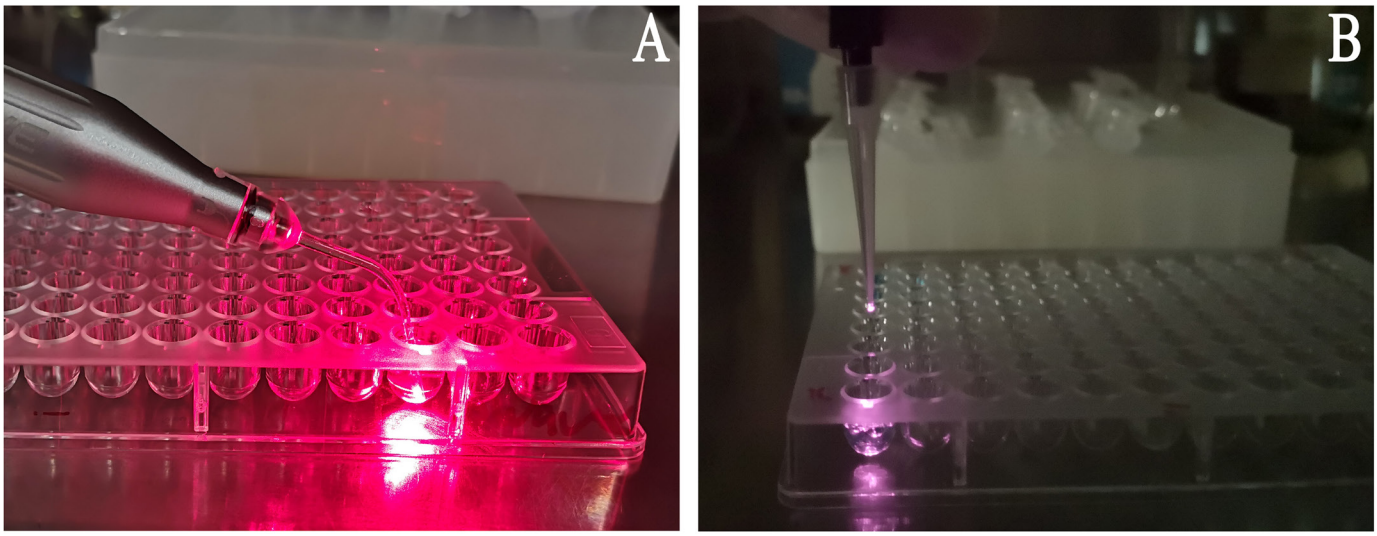

Figure 1. *The ways of the laser irradiation. A. The 670-nm diode laser irradiation. B. The 810-nm diode laser irradiation.*

Table 1. *Study groups.*

Groups	1	2	3	4	5	6	7
Energy Density (J/cm^2) (810-nm diode laser)	120	60	15	5	0	0	120
Time (s)	754	377	94	31	0	0	754
MB (0.0125g/l)	√	√	√	√	√	×	×

MB, methylene blue.

wells of 96-well plates. The experimental well was irradiated with an 810-nm diode laser (Pilot, CAO Group, Inc., USA, 500mW, continuous wave, spot diameter = 2 cm, 5 J/cm^2; 15 J/cm^2; 60 J/cm^2; 120 J/cm^2) or a 670-nm diode laser (Periowave®, HHL-1000, Zhengzhou, China, 200mW, the irradiation times were 0s;31s;94s;377s;754s) with or without MB (0.1 g/L, Zhengzhou, China) photosensitizer. Figure 1 showed the ways of laser irradiation. All experiments were carried out in a dark environment.

The study groups are shown in Table 1:

Groups 1–5: 100 µL fungal suspension + 75 µL saline + 25 µL MB. (Final MB concentration:0.0125g/l)

Groups 6–7: 100 µL fungal suspension + 100 µL saline.

In the process of laser irradiation, an infrared thermometer (Tianjin Key Laboratory of Laser Medicine, Tianjin, China) was used to measure the temperature of the experimental well every 10s.

After laser irradiation, 10 µL of fungal suspension was taken from the experimental well and put into 990ml saline (diluted 100-fold). Then, 100 µL of the diluent was plated on Sabouraud dextrose agar and the number of colony-forming units (CFU) was counted using Image J software after 24h of incubation at 37°C. Then, 1 McFarland turbidity of fungal suspension was prepared to perform drug susceptibility tests. Each experimental group was tested three times. The fungal reduction and antifungal effect of photodynamic therapy = 1 – CFU (after irradiation)/CFU (without irradiation).

After laser irradiation, another 50 µL of the fungal suspension was taken from the experimental well and placed on a slide, and the morphology of *C. albicans* was observed under a microscope. The susceptibility profiles of C.albicans isolates with phenotypic resistance to fluconazole were tested using the standardized colorimetric microdilution test method, Sensititre YeastOne (Thermo Fisher Scientific).

Data were analyzed using SPSS software v.20, t-test, and one-way analysis of variance.

Results

The fungicidal effects of the two kinds of lasers:

Table 2 and Figure 2 show the fungal reduction and antifungal effect of photodynamic therapy of the two lasers. In the presence of the photosensitizer (MB), both lasers had excellent fungicidal effects against the standard (susceptible) and fluconazole-resistant *C. albicans* strains. The fungal reduction and antifungal effect of photodynamic therapy of the 670-nm diode laser was better than that of the 810-nm laser. With or without MB, table 3 showed that there was no significant difference in fungal reduction and antifungal effect of photodynamic therapy between fluconazole-resistant *C. albicans* and the standard strain.

Effect of laser irradiation on temperature:

Figure 3 showed the fungal reduction and antifungal effect of photodynamic therapy resulted in an increase in temperature of <2°C

The laser exposure could not change the drug resistance of the fluconazole-resistant *C. albicans.*

The minimum inhibitory concentration (MIC) of the fluconazole-resistant *C. albicans* was>256ug/ml. And the laser irradiation did not change the MIC.

Table 2. *Fungal reduction and antifungal effect of photodynamic therapy of the 810-nm and 670-nm diode lasers.*

	Fluconazole -Resistant	Time (s)	CFU (Mean)	SD	Fungal Reduction and Antifungal Affect of Photodynamic Therapy (%)
810nm+MB.	Y	0	1735	91.528	
		31	1887	226.930	-8.760
		94	1630	189.241	6.051
		377	756	33.407	56.427***
		754	456	32.187	73.718***
	N	0	1366	158	
		31	1413	112.095	-3.441
		94	1262	140.897	7.613
		377	535	47.149	60.835**
		754	341	33.081	75.037**
	Y	0	1665	81.132	
		754	1749	59.573	-5.050
	N	0	1265	85.712	
		754	1325	101.933	-4.743
670nm+MB	Y	0	1566	121.167	
		31	847	168.075	45.913*
		94	102	30.610	93.487**
		377	2	1	99.872**
		754	0	0.577	100**
	N	0	1168	61.760	
		31	495	67.988	57.620**
		94	38	12.490	96.747***
		377	0	0.577	100***
		754	1	1.155	99.914***
670nm-MB	Y	0	1744	64.902	
		754	1340	36.346	23.165*
	N	0	1265	101.933	
		754	947	90.163	25.138

CFU, colony-forming units; SD, standard deviation; Y, Yes; N, No.
**P < 0.05; **P < 0.01; ***P< 0.001.*

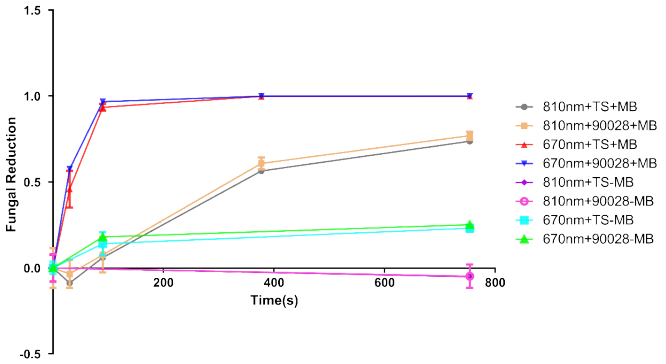

Figure 2. *The fungal reduction and antifungal effect of photodynamic therapy of 810-nm and 670-nm diode lasers.*

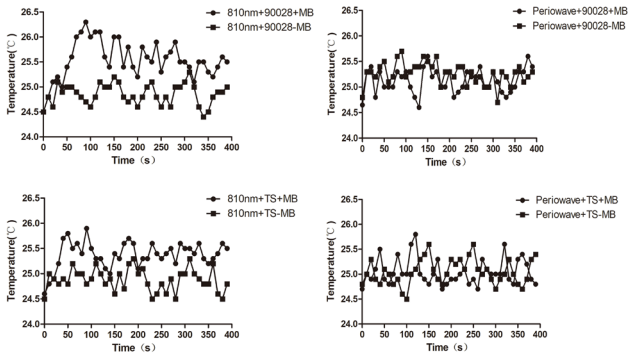

Figure 3. *Temperature changes of experimental wells during laser irradiation was within 2°C.*

Table 3. *There was no significant difference in fungal reduction and antifungal effect of photodynamic therapy between the two strains.*

	Time (s)	*Fluconazole -Resistant*	*Fungal Reduction and Antifungal Effect of Photodynamic Therapy(%)(Mean)*	*F*	*P*
810nm+MB	31	Y	-8.780	0.355	0.583
		N	-3.465		
	94	Y	6.052	0.032	0.866
		N	7.613		
	377	Y	56.427	3.732	0.126
		N	60.835		
	754	Y	77.038	3.553	0.133
		N	73.718		
810nm-MB	754	Y	-5.045	0.004	0.953
		N	-4.769		
670nm+MB	31	Y	45.891	2.755	0.172
		N	57.591		
	94	Y	93.487	6.422	0.064
		N	96.746		
	377	Y	99.872	4.525	0.101
		N	99.971		
	754	Y	99.979	0.354	0.588
		N	99.943		
670nm-MB	754	Y	23.165	0.206	0.673
		N	25.112		

CFU, colony-forming units; Y, Yes; N, No.
**P < 0.05; **P < 0.01; ***P < 0.001.*

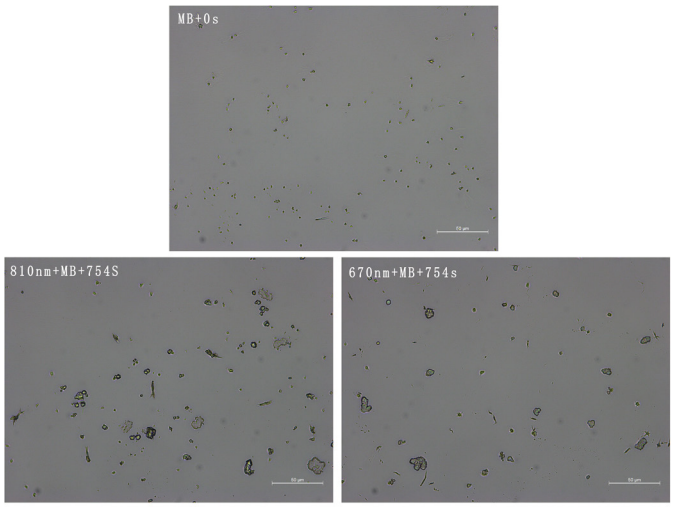

Figure 4. *C.albicans expanded and lysed after laser irradiation (×400 magnification). A. MB+0s (The control group). B. 810nm+TS+MB (754s). C.670nm+TS+MB (754s).*

Morphological changes of the two kinds of *C. albicans* after laser irradiation:

Microscopic analysis indicated that laser-irradiated *C. albicans* expanded and lysed in Figure 4, which may explain how the *C. albicans* die.

Discussion

Candidiasis is a global problem. Many previous studies had looked at the PDT as an alternative to antifungal therapy with remarkable results [13,15]. There are many types of photosensitizers for photodynamic therapy: methylene blue (MB), in-docyanine green (ICG), malachite green (MG) , and toluidine blue (TB). And the most common photosensitizer used for antifungal therapy is MB. Ferreira et al' study showed that 660 nm diode laser (690 mW, CW) could completely eliminate fungi with the energy density of 60 J/cm^2, which meant the fungal reduction and antifungal effect of photodynamic therapy reached 100% [11]. This result is similar to our findings, in the presence of MB, the fungal reduction and antifungal effect of photodynamic therapy of the 670-nm diode laser was almost 100% after irradiation for 377s. However, in another similar study, the PDT (660 nm, 30 mW, CW, 9 J/cm^2, 18 J/cm^2 and 27 J/cm^2) fail to eliminate the fungi completely. But the PDT could cause significant reduction of the number of the CFUs of *C. albicans*. The reason why the fungicidal efficiency could not

reach 100% may be that the laser energy was not enough [15].

In addition, other photosensitizers have also been studied. Azizi et al. studied the effect of 808nm and 660nm laser on fungi with different photosensitizers. They suggested that the photosensitizer could significantly improve the fungicidal effect of laser and the laser application (808 nm, 100 Hz PRR) plus ICG caused the most significant reductionin of *C. albicans* CFUs in their vitro research. Although in this study, the fungal reduction and antifungal effect of photodynamic therapy did not achieve 100%, but it proposed another effective photosensitizer, ICG [16]. Fekrazad et al used InGaAIP laser combined with two photosensitizers (phenothiazine dye (new MB) and indocyanine green (ICG; EmunDo®)) to evaluate the fungicidal efficiency of PDT on *C. albicans*. They concluded that the PDT could significantly killed *C. albicans*, but the fungicidal efficiency was independent of the type of photosensitizer [17]. However, Franak Daliri et al suggested that the type of photosensitizer and laser affected the efficiency of PDT [18]. Our study evaluated the PDT efficacy of the two kinds of lasers on two *C. albicans* strains by means of the photosensitizer (MB). The results were consistent with those of Franak Daliri' study, which showed that the type of laser could affect the fungicidal effect of PDT significantly. When the photosensitizer was MB, the fungicidal efficiency of 670nm diode laser is better than 810nm (Table 2).

Previous studies involving *C. albicans* have shown that lasers not only have excellent fungal reduction and antifungal effect of photodynamic therapy [14,19], but can also promote wound healing and recovery [20]. However, few studies have focused on drug-resistant *C. albicans* strains. In the present study, the two types of laser had significant fungal reduction and antifungal effect of photodynamic therapy against both the standard (drug-susceptible) strain and a fluconazole-resistant strain. The laser treatment did not change the MIC of fluconazole-resistant *C. albicans*. Our findings are consistent with the conclusions of other studies, which showed that PDT using a 660-nm laser reduced the survival ability and sensitivity to drug of fluconazole-resistant strains, but did not change their adhesion, their ability to form biofilms, the sensitivity of the biofilms to drugs, or production of various enzymes [21,22].

We observed the changes of temperature and morphology of *C. albicans* during the irradiation process. The PDT caused an increase in the temperature of fungal suspensions (0–377 s), but this was <2°C. This result indicated that the laser could be safely applied in clinical treatment without causing thermal damage to tissues. Through microscopic observation, we found that the PDT caused expansion and lysis of *C. albicans* cells. Hees and Zhang' experiments also showed that when the energy of irradiation reached a certain level, the cytoderm of *C. albicans* broke down, the cytoplasm flowed out, and the fungus died [23,24].

Conclusions

In our *in vitro* study, PDT using 810-nm and 670-nm diode lasers had an excellent sterilization effect on fluconazole-resistant and non-resistant strains of *C. albicans*. Use of the 670-nm diode laser with irradiation for 377s in the presence of MB was highly effective..

Declarations

Conflict of interest

The authors declare no competing interests.

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